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Molecular characterization of tannery effluent treating *Pseudomonas* species by 16s rRNA analysis

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ABSTRACT

Effluents from tannery industries contain various chemicals like salts and heavy metals in higher concentrations and are to be reduced to permissible limits before their disposal in to environment. In this study, the effluents of tannery industries were collected for estimating the strength of various parameters. These strengths are treated by biological methods using native effective bacteria isolated from sludge. Ten different genera of bacteria were isolated and tested for bioremediation of effluent. The result was that the bacteria had reduced the concentration of salts in the effluent samples below permissible limits. Among ten isolates, three bacterial isolates had shown greater efficiency in reducing various chemical contents present in tannery effluent. The rates of BOD in the treated samples were 14.26 mg/L, 11.36 mg/L and 8.04 mg/L which showed reduction in BOD at the rate of 87.6%. Similarly, the rates of TDS in these samples were 9408.00 mg/L, 9132.00 mg/L and 868.20 mg/L with a maximum reduction percentage of 90.8. They had also shown reduction of various chemicals and salts below permissible limits. Polymerase Chain Reaction (PCR) was done to investigate the genes responsible for the metal reduction by gram negative bacterium, *Pseudomonas* sp. that had degraded Na, Cr, Ca, Mg and P. The genes that are responsible for degrading salts and metals were shown to be present in these bacteria by using PCR.

KEYWORDS

BOD;
TDS;
PCR;
16s rRNA.

INTRODUCTION

Environmental pollution has become a global concern. The toxic pollutants include acids, alkalies, oils, fats, floating organic dissolved matter and colouring agents. There are various industries such as tannery, paper and pulp, sago, sugar, distillery etc which contribute to this pollution. The disposal of waste waters is of widespread national concern. Industrial activities generate a large number and variety of waste waters which are generally discharged into water streams. The nature of industrial wastes depends upon the industrial processes in which they originate. The problem of adequately handling industrial waste water is more complex and much more difficult than sewage.

Tanning is one of the major industries in our coun-

try. There are about 3000 major tanneries in India. Approximately 314 million kilograms of skin are processed annually. The tanneries discharge 3000 litres of waste water, 100 kg-1 of processed hides and the annual discharge of 9420 kilolitres. Tannery industry is reputed globally as a major industry, which contributes to water pollution, owing to the usage of mineral tanning agents. They discharge large volumes of effluents, because except one or two process in the tannery industry, all the processes are wet processes and generate huge quantities of liquid wastes. The effluents are far from the desired level for acceptance into two ways with a heavy load of pollutants like chromium, chlorides, sodium, dissolved solids, BOD, COD, Nitrogen and suspended solids.

Tannery effluents containing large amount of wastes especially tannins are toxic to plants, animals and soil as well as water microorganisms. In plants they cause stunting growth, chlorosis and reduction in yield. However, a few microorganisms degrade tannins and utilize their carbon source. *Chaetomium globosum, Chaetomium cupreum, Fusarium solani, Aspergillus niger* and *Trichoderma viridae* utilizes tannins as carbon source. Species of *Rhizobium, Pseudomonas putida, Pseudomonas solanacearum* grow luxuriantly when cultured in tannin medium.

Tannery effluents from leather tanning industries are major waste water containing high concentration of salts and heavy metals. High salinity in these waste waters poses a major problem in biological effluent treatment process. Moreover, the presence of various toxic heavy metals in tannery effluent causes surface and ground water contaminations and makes them unsuitable for consumption and irrigation. This study involves collecting of effluent samples from tannery effluent treatment plant and treating with bacteria that were isolated from sludge. The samples are treated with bacteria that were identified and later grown with media containing heavy metals. The samples were then incubated for two weeks. At the end of second week, the samples were tested for reduction of various parameters. The aim of the this study was to isolate heavy metal degrading bacteria from tannery effluent samples collected from a tannery industry located in Dindigul (TN), India and to study their mechanism by using Polymerase Chain Reaction (PCR) technique.

The polymerase chain reaction (PCR) is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations. In the first step, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting. In the second step, the temperature is lowered and the two DNA strands become templates for DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers (here *chr* B is used as primer) that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

MATERIALS AND METHODS

Sample collection and bacterial isolation

The tannery effluents and sludge were obtained from outlet of tannery effluent treatment plant which is situated at Dindigul in Tamil Nadu. Bacteria were developed to resist the impact of heavy metals in the effluent. Nutrient agar is a microbiological growth medium commonly used for the routine cultivation of non-fastidious bacteria. This medium is incorporated with heavy metals like Fe, Cu and Ni. The concentration of the metal was maintained at 50µg/ml of the medium. The tannery effluent bacteria were directly inoculated on nutrient media and incubated for 24 hours at 37°C. The plates were observed after the completion of incubation period. For further studies, ten isolates were selected based on their growth on agar media containing heavy metals.

A numerous tests like microscopic tests, biochemical tests were made on the isolates according to Bergery's manual of systemic bacteriology. The specific kind of bacterial species that resist the metals were determined by standard quantitative analyses. Then the selected isolates were stocked up on nutrient agar slants at 4°C for further studies.

Analysis physico-chemical characteristics of tannery effluents

Physicochemical parameters of tannery effluents such as determination of pH, electrical conductivity, estimation of total alkalinity, estimation of acidity and total hardness (including calcium, magnesium, chloride and fluoride). The estimation of salts like nitrate, ammonia, manganese, sulphate, inorganic phosphorous, estimation of potassium and sodium present in the effluents were done using various methods.

Tandardisation of bacterial growth

The bacterial isolates were grown in nutrient broth and the growth was estimated by using spectrophotometer at 600nm. The log phase of growth of isolates was used for effective degradation of tannery effluent salts.

Determination of electrical conductivity

The conductivity cell was rinsed thoroughly with deionized water (the conductivity of the deionized water should be less than 1 μohms/cm). The cell was rinsed with one or more portion of the sample. The temperature of the sample was noted down and multiplied with the correction factor to measure conductivity at 25^0C ± 0.1^0C.

Estimation of biological oxygen demand (BOD)

A desired volume of distilled water with 1 ml/L of phosphate buffer, $MgSO_4$, $CaCl_2$ and $FeCl_3$ were mixed and aerated for 30 minutes. This is known as dilution water. The samples were diluted using standard dilution tables and dilution water. Two sets of BOD bottles were filled with respective solutions of dilution range. One bottle was incubated with label at 20^0C in a BOD incubator for 5 days. Initial DO level was estimated in the other set of bottle and noted down. After 5 days, final DO was estimated and the BOD was calculated using the standard formula.

Estimation of chemical oxygen demand (COD)

20ml of the sample was taken on a round bottomed flask and a pinch of mercuric sulphate was added. Antidumping granules were added. 5ml of sulphuric acid-silver sulphate mixture was added to solution in flask and mixed well to facilitate dissolution of mercuric sulphate. 10ml of potassium dichromate was added and 25 ml of sulphuric acid-silver sulphate mixture was added. While adding this mixture, the round-bottom flash should be kept in ice water bath. This was to prevent the escape of fatty acids due to higher temperature. Then round bottom flask was connected to reflux condenser and refluxed for 2 hours.

After 2 hours the flask was cooled and 80 ml of distilled water was added and mixed well. This was titrated against FAS solution using ferroin indicator. The end point was the sharp color change from blue green to wine red. Simultaneously, the blank was refluxed in the same manner using distilled water instead of sample with the same amount of chemicals.

Estimation of total alkalinity

50 ml of sample was taken in a conical flask and 1-2 drops of phenolphthalein indicator was added. It was titrated against 0.1N HCl until disappearance of pink color. From volume of HCl added, phenolphthalein alkalinity was calculated. After completion of first titration, 2-3 drops of methyl orange indicator was added to conical flask and titration was continued until color change from orange to pink. The total alkalinity was calculated from total volume of HCl consumed in both titrations.

Estimation of sodium and potassium

Flame photometer was switched on as per instructions given in user's manual. The standard solutions were aspirated into flame and instrument was calibrated. The sample was filtered using a filter paper to remove suspended matter which will otherwise clog capillary of instrument. The filtered sample was inspirited into flame and concentrations of sodium and potassium present in sample were determined.

Estimation of hardness

20ml of the sample was taken in a conical flask. To this 2ml of buffer solution and a spatula of Eriochrome

Black-T were added. It was titrated against EDTA solution taken in a burette. The end point was color change from wine red to blue.

Estimation of calcium hardness

20ml of the sample was taken in a conical flask. To this 2 ml of NaOH solution and a spatula of Calcon indicator was added. It was titrated against EDTA solution. The end point was color change from pink to purple.

Estimation of chlorides

20ml of the sample was taken in a conical flask. Few drops of potassium chromate was added and titrated against standard $AgNO_3$ (0.014N). The end point was color change from yellow to brick red. The flask was shaken vigorously during titration to avoid clumping of precipitate.

Bacterial genome isolation and gel electrophoresis

1 ml of culture which contains 1x 10^9 bacteria was collected in a 2ml microcentrifuge tube. It was spun at 12000xg for 30 seconds. The supernatant was discarded and the bacterial pellet was resuspended in 150µl of Buffer S, containing RNase A (50 mg/ml). 20µl of lysozyme was added and mixed well. It was allowed to stand at room temperature for 5min. before proceeding. 30µl of 0.25M EDTA (PH8.0) was added, mixed well and incubated on ice for 5minutes. 450µl of Buffer G-A was added and vortexed for 15 seconds. It was heated in a water bath at 65° C for 10 min. 400µl of Buffer G-B was added, followed by 1ml of Buffer DV (pre-chilled to 4°C) and mixed vigorously. It was centrifuged at 12000xg for 2min. Aspirated off as much of the upper phase as possible without disturbing the interphase upper phase discarded. 1ml of Buffer DV (prechilled to 4°C) was added to the remaining interphase and lower phase and mixed vigorously to achieve homogeneity and centrifuged at 12000xg for 2 min. The colored upper phase was discarded. The lower phase was transferred to a Spin –filter placed in to a 2ml microfuge tube and centrifuged at 12000xg for 1min. The Spin-filter was discarded. 400µl of buffer BV was added to the filtrate and mixed well. A Miniprep column was placed to a 2ml microcentrifuge tube. The binding mix from the above step was transferred to the Miniprep column and centrifuged at 12000xg for 1min. The filtrate from the 2ml microcentrifuge tube was discarded. The Miniprep column was placed back to the 2ml microcentrifuge tube. 500µl of Buffer W1 was added to the Miniprep column and centrifuged at 12000xg for 1min. The filtrate was discarded and the Miniprep column was placed back to the 2ml microcentrifuge tube. 700µl of Buffer W2 was added and centrifuged at 12000xg for 1min. This wash step was repeated with a second 700µl aliquot of Buffer W2. The filtrate was discarded. The Miniprep column was placed back in to the 2ml microcentrifuge tube and centrifuged at 12000xg for 1min. The Miniprep column was transferred to a clean 1.5ml Microcentrifuge tube. To elute the DNA, 100-200µl of Eluent was added to the center of the membrane and was allowed to stand for 1minute at room temperature. It was centrifuged at 12000xg for 1min. The filtrate which contains bacterial genomic DNA was collected and stored at 4°C.

Amplified products were quantified using 1% agarose gel electrophoresis. 0.5g of agarose powder was dissolved in 50 ml TAE buffer (make up to 1 X) and kept in microwave oven for 2 min. 2µl of Ethidium bromide is added and mixed well until it reaches a warm temperature. The prepared agarose was casted on gel tray by placing the comb. 1 µl of 6X Gel loading dye, 2µl of distilled water and 3µl of template (for bacterial genomic DNA) was mixed and loaded in the wells. 6ul of PCR product are loaded onto the well directly without adding gel loading dye. The sample was run at 100 Volts for 30 min.

Quantification of bacterial genome

Psedomonas species DNA was quantified using Hybrid reader.

Polymerase Chain Reaction (PCR) analysis of 16s rRNA

The PCR reaction was performed using 50.0 ng (1.0 µl) of Template (DNA) and 10 pmol (each 2 µl) of primer (forward and reverse separately), and 10 µl PCR Master mix (Amplicon) and 5.0 µl of double-distilled water to adjust the volume to 20.0 µl and the amplification conditions used were as follows:

Primer sequence of 16 sRNA

Gene	Forward primer	Reverse primer
16 sRNA	AGA GTT TGA TCM TGG CTC AG	TAC GGY TAC CTT GTT ACG ACT T
Bacteria (*Pseudomonas*)	GTCGTTAGCTTGCCAACATC	CGGAAAGCAAGATGTCGATCG

RESULTS AND DISCUSSION

Sample collection and bacterial isolation

Ten isolates were isolated from the tannery effluent and sludge. They were identified and characterized by various biochemical tests. The results were tabulated in

TABLE 1 : Identification of bacterial isolates isolated from tannery effluent and sludge

S. No.	Name of isolates	Name of bacteria
1	TEB 1	*Arthrobacter*sp.
2	TEB 2	*Bacillus* sp.
3	TEB 3	*Pseudomonas*
4	TEB 4	*Escherichia coli*
5	TEB 5	*Chromohalobacillus*
6	TEB 6	*Bacillus subtilis*
7	TEB 7	*Pseudomonas aeruginosa*
8	TEB 8	*Staphylococcus aureus*
9	TEB 9	*Bacillus flexus*
10	TEB 10	*Halobacteriumhalobium*

TABLE 1.

Physico-chemical characteristics of tannery effluent

Raw tannery effluents containing metals and vari-

TABLE 2 : Physico-chemical properties of raw tannery effluent and BIS recommended permissible limits for treated tannery effluent

Sl. No.	Parameter (mg/L)	Untreated effluent (raw)	Permissible limits for tannery effluent (treated)
1.	Sodium	1646	Not Mentioned
2.	Potassium	654	Not Mentioned
3.	Calcium	1302	75
4.	Magnesium	486	50
5.	Chloride	7277	1000
6.	Phosphorus	4	Not Mentioned
7.	Nitrate	72	100
8.	Nitrite	56	Not Mentioned
9.	Sulphate	24	400
10.	Fluoride	26	1.5

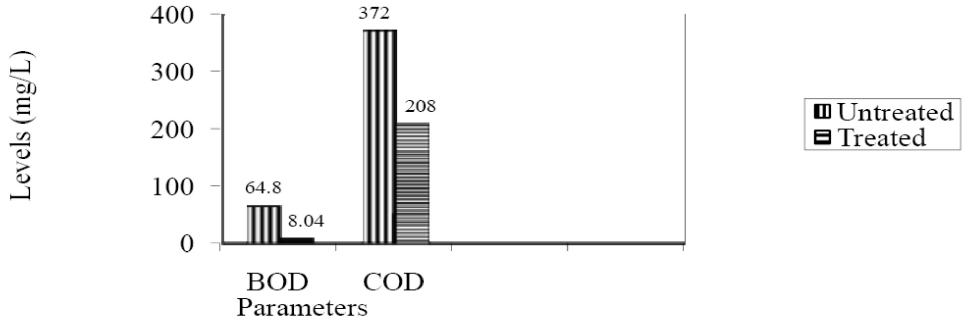

Figure 1: Comparison of BOD and COD of untreated and treated tannery effluent

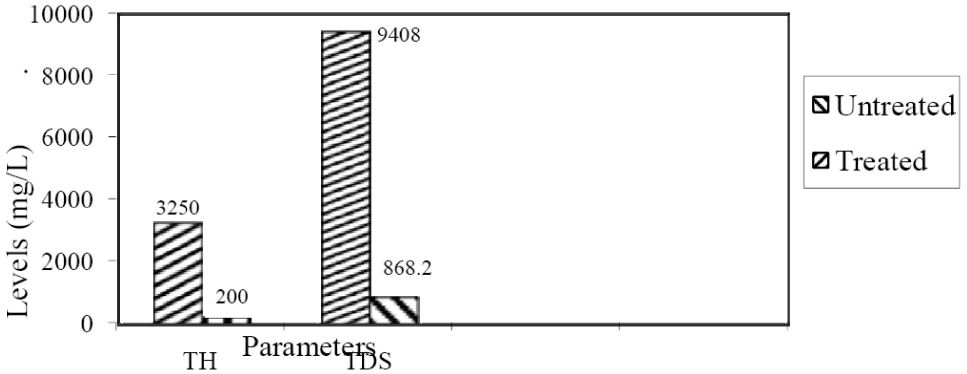

Figure 2 : Comparison of TH and TDS of untreated and treated tannery effluent

ous salts in higher amount and are not suitable for further use like irrigation. Hence, chemicals like sodium, calcium, chloride and various salts must be reduced at maximum levelbefore disposal. The main chemicals present in tannery effluent are cadmium, sodium, potassium and calcium. Moreover, raw tannery effluent has greater electrical conductivity, BOD, COD, TDS and TSS (TABLE 2 and Figures 1 and 2). These parameters are also considered during biological treatment of tannery effluent.

TABLE 3 : Comparison of physico-chemical properties of untreated and treated tannery effluents

Sl. No.	Parameter	Untreated effluent (raw)	Treated effluent
1	Electrical conductivity (µmho/cm)	15860	1447
2	Biological Oxygen Demand (mg/l)	64.80	8.04
3	Chemical Oxygen Demand (mg/l)	372	208
4	Total Dissolved Solids (mg/l)	9408	868.2
5	Total hardness (mg/l)	3250	200.0
6	Sodium mg/l	1640.0	689.0
7	Potassium mg/l	654.0	315.0
8	Calcium mg/l	1302.0	80.0
9	Magnesium mg/l	486.0	36.0
10	Chloride mg/l	7277	2840
11	Phosphorous mg/l	4.0	2.60
12	Nitrate mg/l	72.0	45.0
13	Nitrite mg/l	56.0	32.0
14	Sulphate mg/l	24.0	16.5
15	Fluoride mg/l	26.0	16.0

TABLE 4 : Comparison of parameters of untreated and treated tannery effluents

Sl. No.	Parameter	Raw	TEB 2	TEB 4	TEB 6
1	EC µmho/cm	15860	1568	1522	1447
2	BOD mg/l	64.80	14.26	11.36	8.04
3	COD mg/l	372.0	256.0	218.0	208.0
4	TDS mg/l	9408	940.80	913.20	868.20
5	Sodium mg/l	1640.0	717.0	713.0	689.0
6	Potassium mg/l	654.0	336.0	324.0	315.0
7	Total Hardness mg/l	3250.0	325.0	250.0	200.0
8	Calcium mg/l	1302.0	130.0	100.0	80.0
9	Magnesium mg/l	486.0	48.0	42.0	36.0
10	Chloride mg/l	7277	3550	3260	2840
11	Phosphorous mg/l	4.0	3.10	2.80	2.60
12	Nitrate mg/l	72.0	55.0	50.0	45.0
13	Nitrite mg/l	56.0	40.0	36.0	32.0
14	Sulphate mg/l	24.0	20.0	17.5	16.5
15	Fluoride mg/l	26.0	20.0	18.0	16.0
16	Chromium mg/l	0.55	0.026	0.024	0.021

Comparison of physico-chemical properties of

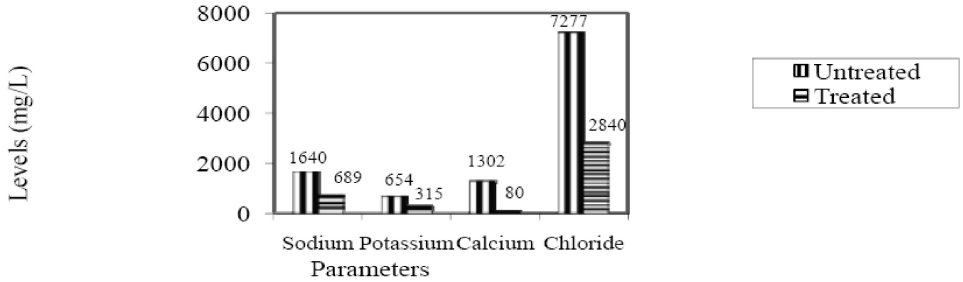

Figure 3 : Comparison of Sodium, Potassium, Calcium and Chloride of untreated and treated tannery effluent

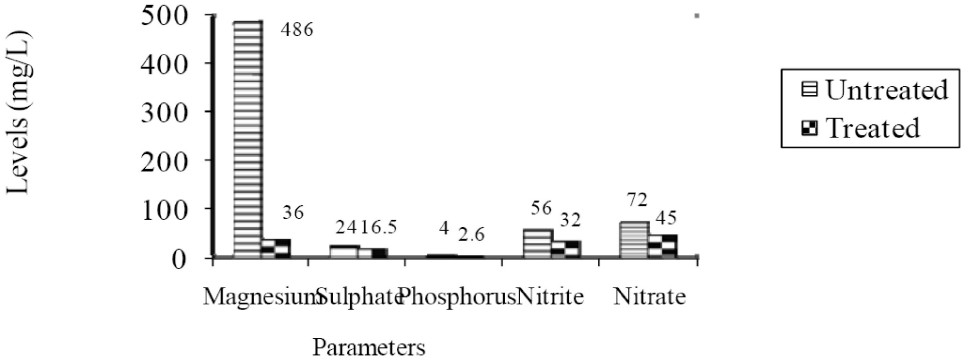

Figure 4 : Comparison of Magnesium, Sulphate, Nitrite and Nitrate of untreated and treated effluent

treated and untreated tannery effluent

Bioremediation process is used for stimulating the microorganisms to grow rapidly for degrading hazardous organic pollutants in surface and ground water bodies. This process results in rapid increase of microorganism that yields reduction of concentration levels of chemicals in effluent water. Vasanthy (2004) found that bacteria like *Pseudomonas* species play a major role in reduction of metals in tannery effluent. The bacteria like *Halobacillus, Chromohalobacillus* and *Pseudomonas* species were used to deduce the concentration level of metals in tannery effluent (TABLE 3 and Figures 3 and 4).

TABLE 5 : Comparison of percentage of reduction (%) of parameters of untreated and treated tannery effluents

Sl. No.	Parameters	Percentage of Reduction (%)		
		TEB 2	TEB 4	TEB6
1	EC µmho/cm	90.11	90.4	90.87
2	BOD mg/l	77.99	82.46	87.5
3	COD mg/l	31.18	41.39	44.08
4	TDS mg/l	90	90.29	90.77
5	Sodium mg/l	56.28	56.52	57.98
6	Potassium mg/l	48.62	50.45	51.83
7	Total Hardness mg/l	90	92.30	93.84
8	Calcium mg/l	90.01	92.31	93.85
9	Magnesium mg/l	90.12	91.35	92.59
10	Chloride mg/l	51.21	55.20	60.97
11	Phosphorous mg/l	22.5	30	35.0
12	Nitrate mg/l	23.61	30.55	37.5
13	Nitrite mg/l	28.57	35.71	42.85
14	Sulphate mg/l	16.66	27.08	31.25
15	Fluoride mg/l	23.07	30.76	38.46
16	Chromium mg/l	95.27	95.63	96.18

Out of three cations tested (Mg, Na, Ca) the calcium level (80mg/l) followed by sodium (689mg/l) and magnesium (36mg/l). The values of potassium and chloride content are 315mg/l and 2840 mg/l respectively but these values obtained after treatment of effluent seems to be higher than BIS standard. The TABLE 3 depicts the reduction of chloride level from 7277 mg/L to 2840 mg/L. Similarly, fluoride content has also reduced from 26 mg/L to 18 mg/L (TABLES 4 and 5 and Figures 5,6,7 and 8).

Bacterial genome isolation and gel electrophoresis

The bacterial genome (DNA) was isolated from *Pseudomonas* species and it was run by gel electrophoresis (Figure9)

Quantification of bacterial genome

DNA was quantified using Hybrid reader and the

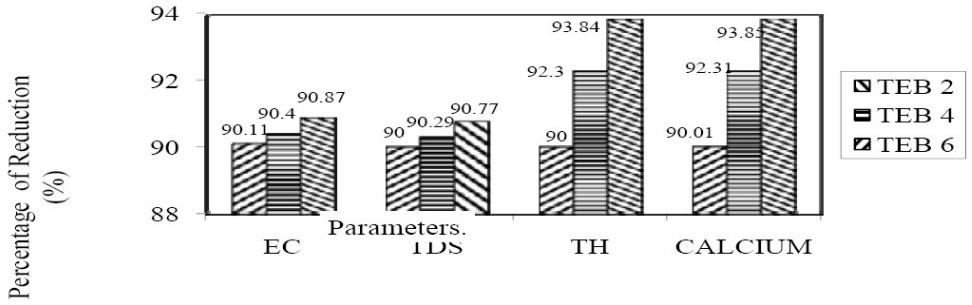

Figure 5 : Percentage of Reduction of Physico-chemical parameters by three bacterial isolates (TEB-2, TEB-4, TEB-6)

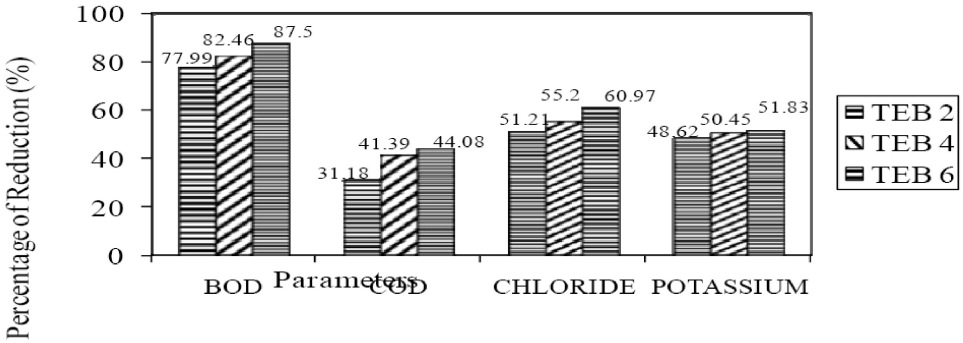

Figure 6 : Percentage of Reduction of Physico-chemical parameters by three bacterial isolates (TEB-2, TEB-4, TEB-6)

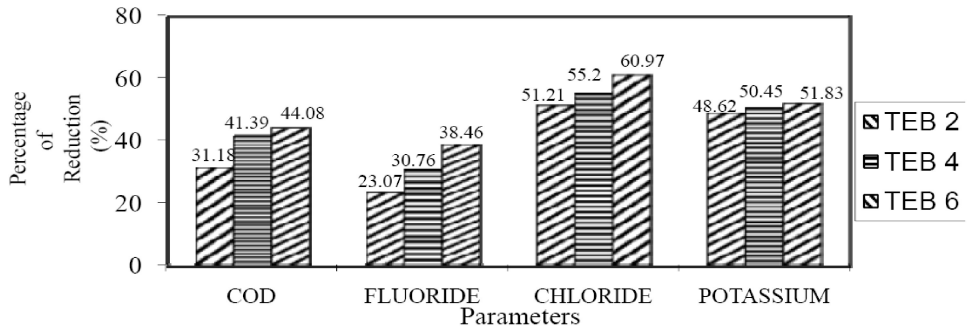

Figure 7 : Percentage of Reduction of Physico-chemical parameters by three bacterial isolates (TEB-2, TEB-4, TEB-6)

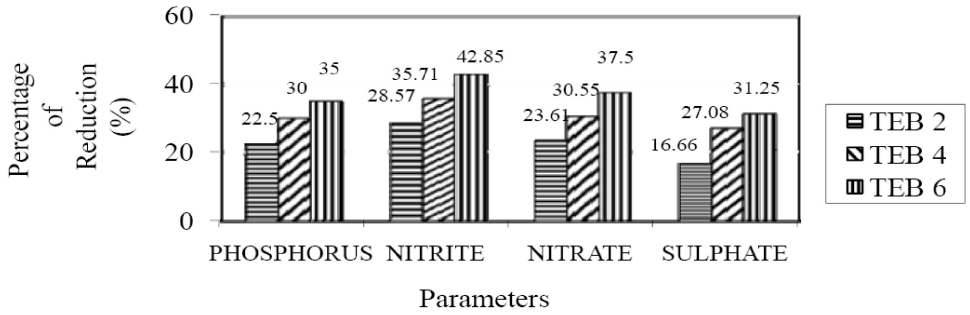

Figure 8 : Percentage of Reduction of Physico-chemical parameters by three bacterial isolates (TEB-2, TEB-4, TEB-6)

Figure 9 : Bacterial genome from *Pseudomonas* species

value (ng/µl) is noted to proceed further to PCR and the results were tabulated in TABLE 6.

TABLE 6 : Quantification of Bacterial DNA

S. No.	Bacteria	Absorbance at 260/280nm	Quantity of bacterial DNA (ng/µl)
	Bacteria (*Pseudomonas*)	1.785	245.764
	Bacteria (*Pseudomonas*)	1.886	240.442

Polymerase Chain Reaction (PCR) analysis of 16s rRNA

In lane 1, molecular ladder was placed and lanes 2 and 3 were 16s rRNA with lanes 4 and 5 were *Pseudomonas* amplicons. From these, the presence of gene for degradation was evident.

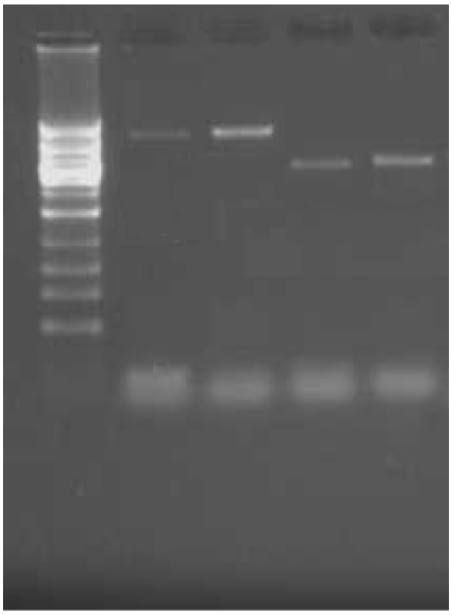

Figure 10 : 16s rRNA of *Pseudomonas* species

DISCUSSION

In this study, three bacterial isolates (*Pseudomonas* species, *Halobacillus* species, *Chromohalobacillus* species) have shown effective degradation of various salts present in the tannery effluent. Out of three one bacterium (*Pseudomonas* species) has shown maximum degradation capacity in all parameters. The bacterium (*Pseudomonas* species) has

reduced TDS (90.7%), hardness (93.8%), BOD (87.5%), COD (44.08%) and various salts. Similar types of results were obtained by previous research (Jatavathu, *et.al.* 2011, Murali, *et.al.*2002). However, the bacterium in this study has shown maximum highest degradation. It was also noted that the genes for degradation were identified.

CONCLUSION

This study offers an alternative to be further exploited for salt and metal bioremediation in a wide range of environments including tannery effluent. The novel finding of chromosome mediated bioremediation of tannery chemicals will attract a considerable interest owing toits potential use in biotechnology, and in creation of more efficient bacteria employing genetic engineering tools and techniques. From PCR and DNA sequence analysis evidence was provided that the loci conferring resistance to these metals are present within gram-negative bacterial communities. Results indicated that the bacterial isolate *Pseudomonas* sp. could be efficiently used for bioremediation tannery effluents. However, results in this study provide a basis for assessing the potential of using indigenous salt and metal reducing novel bacteria for bioremediation applications. Development of economic and efficient bioremediation technique based on whole cell system for the reduction and removal of toxic salts and metals from tannery effluents and solid wastes may help to cope with the extensive use of salt and metal in leather tanning.

LIST OF ABBREVIATION

BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
TH	Total Hardness
TSS	Total Suspended Solids
PCR	Polymerase Chain Reaction
CRD	Chromate Resistant Determinant
DO	Dissolved Oxygen
EDTA	Ethlene Diamine Tetraacetic Acid
DPC	Diphenylcarbazide
LB	Luria Bertani

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